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Abstract: Periodontitis is an inflammatory condition that destroys the tooth supporting tissues, including the alveolar bone. It is triggered by polymicrobial biofilms attaching on tooth surfaces, which can be supragingival or subgingival. Bone resorption is triggered by receptor activator of NF- κ B ligand (RANKL) and blocked by its soluble decoy receptor osteoprotegerin (OPG), which are cytokines of the tumor necrosis factor ligand and receptor families, respectively. The present study aimed to comparatively investigate the effects of the Zürich in vitro supragingival and subgingival biofilm models, on RANKL and OPG gene expression in primary human gingival fibroblasts (GF) cultures. The cells were challenged with biofilm culture supernatants for up-to 24h. RANKL and OPG gene expression in the cells was analyzed by quantitative real-time polymerase chain reaction (qPCR) and their relative RANKL/OPG ratio was calculated. Both biofilm supernatants induced RANKL expression, but the subgingival caused a more pronounced up-regulation compared to the supragingival (10-fold at 6h and 100-fold at 24h). Changes in OPG expression in response to either biofilm were more limited. Accordingly, the subgingival biofilm caused a greater enhancement of the relative RANKL/OPG ratio (4-fold at 6h and 110-fold 24h). In conclusion, subgingival biofilms exhibit a stronger potency for inducing molecular mechanisms of bone resorption than supragingival biofilms, in line with their higher virulence nature for the development of periodontitis.

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The RANKL-OPG system is differentially regulated by supragingival and subgingival biofilm supernatants

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Abbreviations

RANKL: receptor activator of NF- κ B ligand, OPG: osteoprotegerin; GF: gingival
fibroblasts, LDH: lactate dehydrogenase; qPCR: quantitative real-time polymerase
chain reaction

Abstract

Periodontitis is an inflammatory condition that destroys the tooth supporting tissues, including the alveolar bone. It is triggered by polymicrobial biofilms attaching on tooth surfaces, which can be supragingival or subgingival. Bone resorption is triggered by RANKL and blocked by its soluble decoy receptor OPG, which are cytokines of the tumor necrosis factor ligand and receptor families, respectively. The present study aimed to comparatively investigate the effects of the Zürich *in vitro* supragingival and subgingival biofilm models, on RANKL and OPG gene expression in primary human GF cultures. The cells were challenged with the biofilms for up-to 24 hours. RANKL and OPG gene expression in the cells was analysed by qPCR and their relative RANKL/OPG ratio was calculated. Both biofilms induced RANKL expression, but the subgingival caused a more pronounced up-regulation compared to the supragingival (10-fold at 6 h and 100-fold at 24 h). Changes in OPG expression in response to either biofilm were more limited. Accordingly, the subgingival biofilm caused a greater enhancement of the relative RANKL/OPG ratio (4-fold at 6 h and 110-fold 24 h). In conclusion, subgingival biofilms exhibit a stronger potency for inducing molecular mechanisms of bone resorption than supragingival biofilms, in line with their higher virulence nature for the development of periodontitis.

Keywords: Oral biofilms; gingival fibroblasts; receptor activator of NF- κ B ligand; osteoprotegerin; periodontitis

1. Introduction

Periodontal diseases are perhaps the most common chronic inflammatory diseases in man. Their main trait is the inflammatory destruction of the tooth-supporting (periodontal) tissues, as a result of oral bacteria colonizing the tooth surfaces in the form of polymicrobial biofilm communities [1]. Depending on the localization of the biofilm in relation to the gingival margin, this can be either “supragingival” (above) or “subgingival” (below) [2]. Supragingival biofilms are typically constellated by Gram positive, facultative anaerobic and non-motile species, whereas subgingival biofilms are characterized by the dominance of Gram negative, anaerobic and motile species. Bacterial products released by the biofilms can cause an inflammatory response by the periodontal tissues, aiming to eliminate the bacterial challenge [3]. However, rather than being protective, an excessive inflammatory response induces periodontal tissue damage [4]. Gingivitis is a clinical condition in which the host-inflammatory response to the biofilm is restricted to the superficial gingival tissue and is typically associated with the presence of a supragingival biofilm. However, if the inflammation progresses into the deeper periodontal tissues, it is accompanied by a concomitant deepening of the gingival epithelium and the creation of a pathological pouch known as periodontal pocket. The niche of the periodontal pocket is an optimal environment for the colonization and growth of species that preferentially form subgingival biofilm communities [5]. The presence of subgingival biofilms is detrimental for the development of periodontitis, as the associated species are considered more pathogenic for this disease [1, 2, 6, 7]. Periodontitis represents a progressive inflammatory switch from gingivitis that involves further the destruction of the alveolar bone, eventually leading to tooth loss.

Bone resorption in physiological and pathological conditions, such as periodontitis, is regulated by the interplay of a system of two cytokines belonging to the tumor necrosis factor ligand and receptor superfamilies. These are respectively Receptor Activator of NF- κ B Ligand (RANKL) and osteoprotegerin (OPG). RANKL is expressed by osteobasts, synovial or gingival fibroblasts (GF) and activated T- and B-cells. By activating its cognate RANK receptor on osteoclast precursors (cells of the monocyte/macrophage lineage), it triggers their fusion and differentiation into multi-nucleated osteoclasts, which are the bone-resorbing cells [8]. The soluble decoy receptor OPG can bind to RANKL, thus inhibiting the RANK-RANKL interaction and the downstream events that lead to bone resorption [9]. Changes in the relative RANKL/OPG ratio are indicative of the capacity of a cell or tissue to regulate bone resorption. An increased ratio is indicative of enhanced bone resorption in pathological inflammatory conditions, such as periodontitis [10, 11].

The cells of the gingival connective tissue have an important role in the protection and homeostasis of the periodontium. GF constitute the main cell population of this tissue producing collagenous matrix, but also responding to bacterial challenge by producing mediators of inflammation [12]. They constantly express OPG but do not regularly express RANKL, unless bacterially challenged [13-15]. The potential effects of polymicrobial oral biofilm challenge have not been investigated in this respect. It is not known if supragingival and subgingival biofilms have different capacities in regulating the RANKL-OPG system. The present *in vitro* study aims to compare the capacity of a supragingival and a subgingival biofilm to regulate the expression of the RANKL-OPG system in human GF cultures. It is hypothesized that, due to its more virulent nature for the development of periodontitis,

the subgingival biofilm would cause a more potent up-regulation of the RANKL/OPG expression ratio, potentially enhancing bone destruction.

2. Materials and Methods

2.1 *In vitro* biofilm model

The 6-species supragingival Zürich biofilm model [16] used in this study consisted of *Veillonella dispar* ATCC 17748 (OMZ 493), *Fusobacterium nucleatum* KP-F8 (OMZ 598), *Streptococcus oralis* SK248 (OMZ 607), *Actinomyces naeslundii* (OMZ 745), *Streptococcus mutans* UAB159 (OMZ 918) and *Candida albicans* (OMZ 110).

The 10-species subgingival Zürich biofilm model [17] used in this study consisted of *Campylobacter rectus* (OMZ 697), *F. nucleatum* subsp. *vincentii* KP-F2 (OMZ 596), *Porphyromonas gingivalis* ATCC 33277T (OMZ 925), *Prevotella intermedia* ATCC 25611T (OMZ 278), *Tanerella forsythia* OMZ 1047, *Treponema denticola* ATCC 35405T (OMZ 661), *V. dispar* ATCC 17748T (OMZ 493), *A. naeslundii* OMZ 745, *S. intermedius* ATCC 27335 (OMZ 512), and *S. oralis* SK 248 (OMZ 607). Briefly, the supragingival or subgingival biofilms were grown in 24-well cell culture plates on sintered hydroxyapatite discs, resembling natural tooth surfaces, and were pre-conditioned for pellicle formation with human mixed saliva for 4 h. To initiate biofilm formation, hydroxyapatite discs were covered for 16.5 h with 1.6 ml of growth medium consisting of 60% saliva, 10% human serum (pooled from three donors), 30% FUM culture medium [18] and 200 µl of a bacterial cell suspension containing equal volumes and density from each strain. After 16.5 h of anaerobic incubation at 37°C, the inoculum suspension was removed from the discs by 'dip-washing' using forceps, transferred into wells with fresh medium (60% saliva, 10% human serum, 30% FUM), and incubated for further 48 h in anaerobic atmosphere.

During this time-period, the discs were dip-washed 3x and given fresh medium once daily. After a total 64.5 h of incubation, at an advanced stage of biofilm maturation, the culture supernatants were collected, filtered and stored at -80°C. The composition of biofilms on the hydroxyapatite discs at the time of supernatant collection was performed by bacterial culture analysis, as previously described [16, 17], and the respective bacterial counts are provided in Table 1. It is anticipated that supernatants from earlier stages of biofilm culture would be less virulent, or at least less representative an established biofilm stage. The bacterial protein concentration in these supernatants was determined by the BCA Protein Assay (Pierce). For the experiments, these biofilm supernatant preparations were diluted into the final cell culture medium and maintained in the cell culture for up to 24 h [17]. Their concentration is expressed as total protein ($\mu\text{g/ml}$) present in the cell cultures. While total bacterial protein was selected as a calibration measure to compare the effects of the two biofilm supernatants, qualitative analysis of individual proteins is less feasible due to their vast number in this complex mixture.

2.2 Cell cultures

Primary human GF cell lines were established as previously described [13, 15]. Briefly, gingival tissue biopsies used were obtained from healthy young individuals, who had their first premolar removed during the course of orthodontic treatment. Ethical approval was granted by the Human Studies Ethical Committee of Umeå University, Sweden, and informed consent was given by the subject. The cells were passaged and cultured in Minimum Essential Medium Alpha (Gibco), supplemented with 5 % heat-inactivated foetal bovine serum (Sigma), 50 U/ml penicillin, and 50 $\mu\text{g/ml}$ streptomycin (Sigma). For the experiments, GF cells at passage 3 were seeded

at concentration 10×10^3 cells/cm² in antibiotics-free and 5% FBS culture medium, and were allowed to attach for 24 h, maintaining a sub-confluent status. Thereafter, the cells were cultured for 6 h or 24 h in the presence or absence of ascending protein concentrations of biofilm supernatants.

2.3 Cytotoxicity assay

Potential cytotoxic effects of the two biofilms on GF cultures were evaluated by measurement of the extracellularly released cytosolic lactate dehydrogenase (LDH), using the CytoTox96[®] Non-Radioactive Cytotoxicity Assay (Promega). The cultures were exposed to ascending biofilm protein concentrations up-to 300 µg/ml, for 6 h. The cell culture supernatants were collected, and the cell monolayer was lysed. The cell supernatants and lysates were centrifuged at 1000 rpm for 5 min and thereafter transferred into an optically clear 96-well plate (Petra-plastic, Switzerland), followed by addition of reaction solution and incubated for 30 min in the dark. The reaction was then stopped and the absorbance was measured at 490 nm in a BioRad 3550 microplate reader, subtracting background values from all samples.

2.4 RNA extraction and cDNA synthesis

After completion of the experiments, the culture supernatants were removed from the culture and the cell monolayers were washed twice in PBS before being lysed. The collected cell lysate was homogenized with QIAshredder (QIAGEN), and total RNA was extracted by using the RNeasy Mini Kit (QIAGEN), according to the manufacturer's instructions, and the RNA was finally eluted in 50 µl RNase free water and its concentration was measured by a NanoDrop spectrophotometer. One µg of total RNA was then reverse transcribed into single-stranded cDNA by using M-

MLV Reverse Transcriptase, Oligo(dT)₁₅ Primers, and PCR Nucleotide Mix according to the manufacturer's protocol (all from Promega), at 40°C for 60 min, and 70°C for 15 min. The resulting cDNA was stored at -20°C until further use.

2.5 Quantitative real-time Polymerase Chain Reaction (qPCR)

For RANKL and OPG gene expression analyses, qPCR was performed in an ABI Prism 7000 Sequence Detection System and software (Applied Biosystems). 18S rRNA was used as endogenous RNA control in the samples (house-keeping gene). For the amplification reactions, the TaqMan Gene Expression Master Mix and Gene Expression Assay kits from Applied Biosystems were used (assay IDs RANKL: Hs00243522-m1, OPG: Hs00171068-m1, and 18S rRNA: Hs99999901-s1). The standard PCR conditions were 10 min at 95°C, followed 40 cycles at 95°C for 15 seconds and 60°C for 1 min. The expression levels of RANKL and OPG transcripts in each sample were calculated by the comparative Ct method ($2^{-\Delta C_t}$ formula) after normalization to 18S rRNA.

2.6 Statistical analysis

A two-way analysis of variance (ANOVA) was used to analyze the statistical significances of the results, using Bonferroni post-hoc test for comparisons between individual groups. The data were considered significant at $P < 0.05$.

3. Results

The established human GF cell cultures were challenged with ascending concentrations of the supragingival or subgingival biofilm culture supernatants for 6 h and 24 h. The potential cytotoxic effects were investigated by measuring the

extracellularly released LDH in the culture. Neither supragingival nor subgingival biofilm challenge increased toxicity, compared to the control group (Table 2). Hence it was possible to further perform gene expression analyses.

The effect of the two biofilm challenges on RANKL expression in GF was then investigated (Figure 1A). After 6 h, the cells did not express RANKL in the absence of biofilm challenge or with the lowest biofilm supernatant concentrations used (3 µg/ml). RANKL expression was induced with higher biofilm concentrations, both by supragingival or subgingival supernatants. However, there were quantitative differences in the capacity of the two biofilm supernatants to induce RANKL. At concentrations of 30 µg/ml, the subgingival biofilm caused a 2.6-fold greater up-regulation of RANKL compared to the supragingival biofilm, whereas this difference increased to 9.4-fold with the highest concentration used (300 µg/ml). After 24 h of challenge, a further increase of RANKL expression was evident with both biofilms. However, this was only limited by the supragingival biofilm (0.7-fold compared to 6 h) but very pronounced by the subgingival biofilm, which was 18.3-fold compared to 6 h (Figure 1B).

The expression of OPG in response to the two biofilm types after 6 h and 24 h of challenge was then investigated (Figure 2A). OPG was regularly expressed by the cells, and biofilm supernatant concentrations up-to 30 µg/ml did not affect this expression, irrespective of the type of biofilm. However, at 300 µg/ml concentration, OPG expression was up-regulated by the subgingival biofilm and down-regulated by the supragingival biofilm, compared to the unchallenged control. When OPG expression levels were compared between the two biofilm-challenged groups at this concentration (300 µg/ml), there was a 2.4-fold greater difference in favour of the supragingival. However, after 24 h, OPG expression levels were similar in the two

biofilm-challenged groups, and slightly higher (35%-50%) than the control group (Figure 2B).

Changes in the expressions of RANKL and OPG result in an altered relative RANKL/OPG expression ratio. An increase in this measure is indicative of enhanced capacity by the cells to induce osteoclastogenesis and bone resorption. In the present experimental system, both the supragingival and the subgingival biofilm challenge enhanced the RANKL/OPG ratio in GF (Figure 3). However, it was evident that quantitative differences occurred between the two biofilms in this respect. Compared to the supragingival, the subgingival biofilm induced at 6 h a 2.8-fold and 3.9-fold higher RANKL/OPG ratio at concentrations of 30 µg/ml and 300 µg/ml, respectively (Figure 3A). At 24 h, this difference was dramatically enhanced. At concentrations 300 µg/ml, the subgingival biofilm induced a 112-fold greater RANKL/OPG ratio by the cells, compared to the supragingival biofilm (Figure 3B).

4. Discussion

The advantage of studying host-bacteria interactions in polymicrobial biofilm models over planktonic mono-cultures is that a) several bacterial species are represented in a spatial arrangement that resembles their natural habitat when attached on the tooth surface, and b) it allows the inclusion of species associated with health or different stages of disease. An *in vitro* subgingival biofilm model has so far been used to study apoptotic and pro-inflammatory cytokine responses in gingival epithelial cells [17]. The advantage of using biofilm culture supernatants over co-cultures with live biofilms is that it allows for the investigation of concentration-dependent effects, and also the potential cytotoxic effects of live biofilms over prolonged experimental periods can be avoided. Moreover, during periodontal infection the periodontal

connective tissues are likely to be affected by secreted or released bacterial products, rather than being directly exposed to the biofilm mass.

This is the first comparative study of the effects of supragingival versus subgingival oral biofilms on host cells. In particular, the differential effects of these two biofilm supernatant variants were investigated on the expression of the RANKL-OPG system in GF. The findings demonstrate that both biofilm supernatants can regulate RANKL and OPG gene expressions in GF, in line with a recent study demonstrating that viable supragingival biofilms can regulate this system in periodontal ligament and dental pulp cells [19]. However, there are considerable quantitative differences in their regulatory capacity. At an early time point of challenge (6 h), the subgingival biofilm induces an almost 10-fold higher RANKL expression than the supragingival biofilm. This difference is strongly enhanced to 100-fold over a 24 h period of challenge. Individual putative periodontal pathogens, such as *Aggregatibacter actinomycetemcomitans*, *P. gingivalis*, *T. denticola* and *P. intermedia* have been shown to induce RANKL expression in various cell types, and in some cases a temporal enhancement was also demonstrated [13, 15, 20-22]. Of note, *P. gingivalis*, *T. denticola* and *P. intermedia* are also represented in the subgingival biofilm model used in this study, although their relative individual or cumulative contribution to RANKL induction by the biofilm challenge is not clear at this stage. It is also unclear if the observed effects are attributed to the protein content of the biofilm supernatant, or other non-proteinaceous cell wall components or the extrapolymeric matrix itself. Whichever the case, products of the subgingival biofilm appear to be more potent in this respect. The enhanced induction of RANKL expression over time could be attributed to accumulated production of inflammatory

mediators, such as prostaglandin E2, as has been demonstrated in other experimental systems [20, 21, 23, 24].

The biofilm challenge caused an early deregulation of OPG expression by the cells. In particular, the subgingival biofilm caused a 50% up-regulation of OPG expression, compared to the control. This could be an attempt by the cells to counterbalance the simultaneous excessive induction of RANKL. In contrast, the less virulent supragingival biofilm caused an early down-regulation of OPG expression to 60% of the control, which could denote a less pronounced or more finely controlled initiation of molecular events towards bone resorption. Nevertheless, OPG expression levels were equilibrated over 24 h of challenge, and only slightly up-regulated in relation to the control. A temporal deregulation of OPG expression, with resumption of control levels over time, has been previously demonstrated in experimental systems involving challenge with *P. gingivalis* [15, 23].

The relative RANKL/OPG expression ratio is a measure of clinical relevance, its local increase in the tissues is associated with the occurrence of periodontitis [10, 11, 25]. In the present experimental system, the RANKL/OPG ratio was also calculated, as an indicator of the cells' capacity to enhance osteoclastogenesis and bone resorption. The species represented in this supragingival biofilm model have not been investigated in this respect, although *P. gingivalis*, present in the subgingival biofilm, has been shown to enhance the RANKL/OPG ratio in other experimental systems [15, 23]. The results of the present study indicate that in response to biofilm challenge, this ratio follows a similar trend to RANKL induction. Hence, both biofilms variants can up-regulate the RANKL/OPG ratio in GF over time, indicating that the cells attain an enhanced capacity for stimulating bone resorption. However, there are considerable quantitative differences in this regulatory capacity by the two

biofilms. The subgingival biofilm is more potent than the supragingival biofilm in this respect, causing a 4-fold greater RANKL/OPG ratio enhancement at 6 h, and a remarkable 112-fold after 24 h. The higher capacity of the subgingival biofilm to enhance the RANKL/OPG ratio and potentially bone resorption, is well in line with the strong association of subgingival biofilms with periodontitis [1, 2, 6], in which bone destruction is a major histopathological trait [26, 27].

5. Conclusion

This study demonstrates that supragingival and subgingival biofilms can up-regulate the RANKL/OPG ratio in cells of the gingival connective tissue, denoting an enhanced capacity by the cells to stimulate bone resorption. Nevertheless, subgingival biofilms are considerably more potent in this respect, in line with the higher virulence potential of the periodontitis-associated species [7]. This finding could constitute further molecular proof of the clinical concept that subgingival biofilms are more detrimental for the pathogenesis of periodontitis.

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Tables

Table 1. Characterisation of biofilm composition at time of supernatant collection

Subgingival biofilm

<i>A. naeslundii</i> :	4.1 E7 ± 3.0 E7
<i>V. dispar</i> :	4.7 E5 ± 3.1 E5
<i>F. nucleatum</i> :	5.0 E8 ± 8.2 E7
<i>S. intermedius</i> :	3.0 E8 ± 6.6 E7
<i>S. oralis</i> :	1.7 E8 ± 6.7 E7
<i>P. intermedia</i> :	1.6 E7 ± 5.7 E6
<i>C. rectus</i> :	9.5 E7 ± 3.8 E7
<i>P. gingivalis</i> :	3.1 E5 ± 1.4 E5
<i>T. forsythia</i> :	9.5 E6 ± 6.0 E6
<i>T. denticola</i> :	4.3 E7 ± 1.8 E7

Supragingival biofilm

<i>A. naeslundii</i> :	6.4 E6 ± 1.2 E6
<i>V. dispar</i> :	2.3 E8 ± 8.1 E7

F. nucleatum: 1.1 E8± 4.6 E7

S. mutans: 5.2 E7± 6.7 E6

S. oralis: 3.9 E7± 8.7 E6

C. albicans: 2.5 E4± 1.5 E4

Supernatants were collected after 64.5 h of supragingival or subgingival biofilm culture. The composition of the biofilm-associated species on the hydroxyapatite discs was determined by bacterial culture analysis as previously described [16, 17]. The data represents the bacterial mean counts ± SD from triplicate biofilm cultures.

Table 2. Cytotoxicity of supragingival and subgingival biofilm supernatants on GF.

A.		3 µg/ml	30 µg/ml	300 µg/ml
Control	4.6 ± 1.1			
Supragingival		5.3 ± 0.8	4.2 ± 0.4	2.4 ± 1.2
Subgingival		3.0 ± 0.3	5.2 ± 1.8	2.9 ± 2.0
B.		3 µg/ml	30 µg/ml	300 µg/ml
Control	9.1 ± 0.5			
Supragingival		9.2 ± 0.4	8.8 ± 0.3	9.9 ± 1.2
Subgingival		9.8 ± 1.3	9.3 ± 1.6	9.3 ± 0.4

GF cell cultures were challenged with ascending concentrations of supragingival or subgingival biofilm supernatants for 6 h (A) or 24 h (B). Extracellularly released LDH, representing the relative number of dead cells, is expressed as percentage (%)

of total LDH (extracellularly released + intracellular content) in each group. Numbers represent mean percentage values \pm SD of triplicate cell cultures.

Figure Legends

Figure 1

Regulation of RANKL expression in GF in response to oral biofilm supernatant challenge. GF cell cultures were challenged with ascending concentrations of supragingival or subgingival biofilm supernatants for 6 h (A) and 24 h (B). The gene expression levels of RANKL were measured by qPCR analysis, normalized against the expression levels of the 18S rRNA (housekeeping gene). The results are expressed as the $2^{-\Delta CT}$ formula. Bars represent mean values \pm SEM from three independent experiments. The asterisk represents statistically significant difference between the supragingival and subgingival biofilm-challenged groups.

Figure 2

Regulation of OPG expression in GF in response to oral biofilm supernatant challenge. GF cell cultures were challenged with ascending concentrations of supragingival or subgingival biofilm supernatants for 6 h (A) and 24 h (B). The gene expression levels of OPG were measured by qPCR analysis, normalized against the expression levels of the 18S rRNA (housekeeping gene). The results are expressed as the $2^{-\Delta CT}$ formula. Bars represent mean values \pm SEM from three independent experiments. The asterisk represents statistically significant difference between the supragingival and subgingival biofilm-challenged groups.

Figure 3

Regulation of the RANKL/OPG expression ratio in GF, in response to oral biofilm supernatant challenge. GF cell cultures were challenged with ascending concentrations of supragingival or subgingival biofilm supernatants for 6 h (A) and 24 h (B). The relative RANKL/OPG gene expression ratio was calculated based on the RANKL and OPG gene expression values measured by qPCR. Bars represent mean values \pm SEM from three independent experiments. The asterisk represents statistically significant difference between the supragingival and subgingival biofilm-challenged groups.

Figure 1

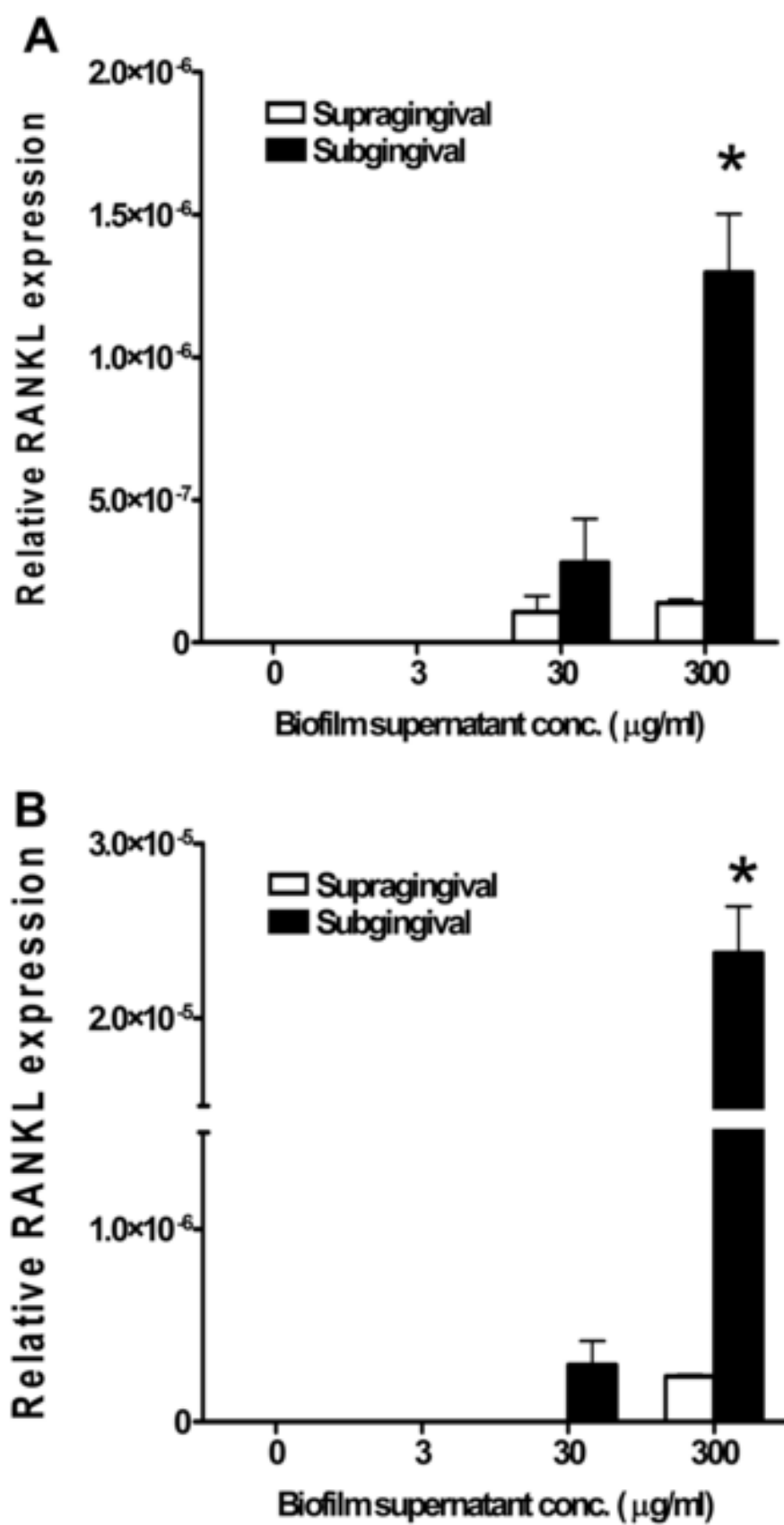


Figure 2

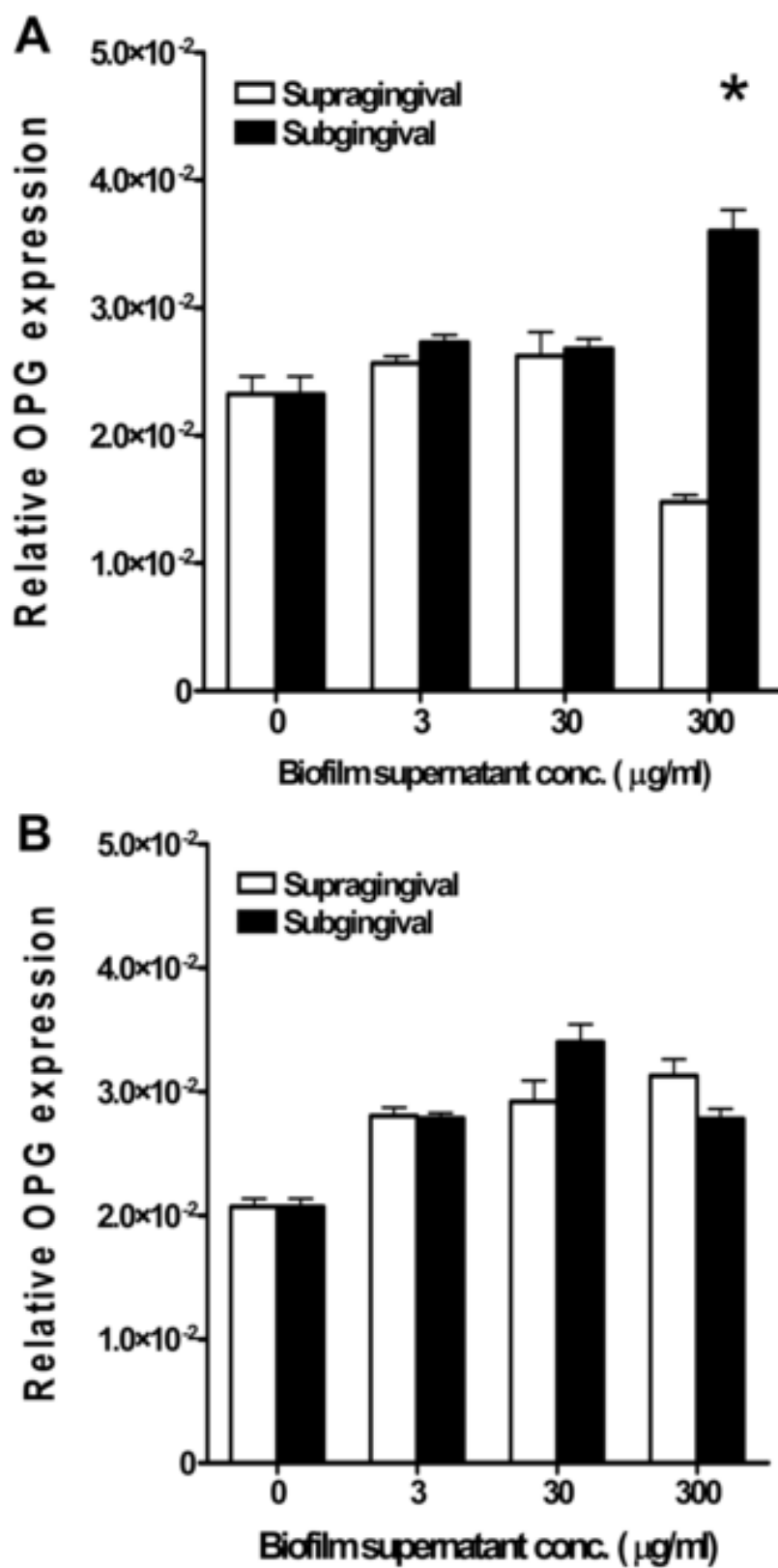


Figure 3

